

# Molecular Diversity Analysis as An Improvement Tool for Pigeonpea [*Cajanus cajan* (L.)]

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## Abstract

Simple sequence repeat (SSR) marker system was used to assess the genetic diversity among forty pigeonpea genotypes using eighty primer pairs. The banding pattern was recorded in the form of 0-1 data sheet which was analyzed using unweighted pair group method with arithmetic mean based on Jaccard's similarity coefficient. The results revealed that out of 80 SSR primers, 65 primers showed distinct polymorphism indicating the robust nature of microsatellites in revealing polymorphism. The number of alleles generated by each marker ranged from 2 to 7, with an average of 3.4 alleles.

The polymorphism information content values for the SSR loci ranged from 0.24 to 0.86. Higher PIC value was observed for SSR primer CZ681974 (0.86) and lowest PIC value (0.24) was observed for the primer CZ682005. The SSR markers showed an average PIC value of 0.50. Markers with PIC values of 0.5 or higher are highly efficient in revealing genetic studies and are extremely valuable in distinguishing the polymorphism rate of a marker at a specific locus. The cluster analysis showed higher level of genetic variation among the genotypes. Similarity coefficients ranged from 0.45 to 0.93.

The dendrogram based on the cluster analysis by microsatellite polymorphism, grouped 40 pigeonpea varieties into 2 major clusters which were further grouped into different sub-clusters. Based on the present study, the large range of similarity values for related genotypes using simple sequence repeats (SSR) provides greater certainty for the evaluation of genetic diversity and relationships for background selections during hybridization based crop improvement programmes.

**Keywords:** *Cajanus cajan*, genetic diversity, simple sequence repeat marker.

## Introduction

Legumes are an integral part of subsistence agriculture since they benefit both humans and plants by providing protein-rich food and nutrition. Pigeonpea [*Cajanus cajan* (L.) Millsp.] also known as red gram, tur, arhar, tuvarica, congobean, gungo pea, no eye pea, thogari or gandul<sup>14</sup> is well adapted to drought conditions and can also be grown on marginal land and has need of limited inputs generally. It is cultivated in approximately 50 countries in Asia, Africa and America. Pigeonpea belongs to the genus *Cajanus* of the subtribe *Cajaninae* under the family Leguminosae. It is the only cultivated food crop of the *Cajaninae* sub-tribe and has a diploid genome with 11 pairs of chromosomes ( $2n = 2x = 22$ ) having 858 Mbp of genome size<sup>7</sup>.

The heritable variation within the organism shows its genetic diversity. It is very important to estimate the genetic diversity from breeding point of view because it provides the basis for selection and is normally measured by genetic distance or genetic similarity. It can be obtained from pedigree analysis, morphological traits or using molecular markers<sup>18</sup>. Genetic diversity is a raw material for evolution permitting populations of species to survive, grow and acclimatize to resist long-term changes in the environment. This is very important in the plant breeding approaches for developing high yielding varieties and maintaining the productivity of such varieties through pyramiding of genes for resistance to disease, insect pests and other abiotic factor<sup>17</sup>.

Most of the characterization and evaluation have been based on the observation of either qualitative or quantitative morphological characters and achieved genetic diversity by statistical advanced methods include; correlation matrix, stepwise regression and cluster analysis. Over the years, the methods for detecting and assessing genetic diversity have been enhanced through the availability of several molecular marker systems. The polymerase chain reaction technology has modernized the field of molecular biology by the introduction of DNA based molecular markers<sup>6</sup>.

Numerous DNA marker systems are currently available to evaluate the variability and diversity studies of plants at

molecular level including Restriction Fragment Length Polymorphisms (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphisms (AFLP), Single Nucleotide Polymorphisms (SNPs), Diversity Arrays Technology (DArT) etc. Statistical information about genetic variability at molecular level can be used to assist, discover and advance genetically unique germplasm that complements existing cultivars.

Out of the wide arrays of DNA markers available, it has been observed that microsatellite or simple sequence repeat (SSR) markers are considered to be suitable for estimation of genetic diversity and variety recognition because of their competence to detect large numbers of discrete alleles repeatedly, perfectly and efficiently<sup>8,23</sup>. SSRs are short stretches of tandemly repeated, 1 to 6 nucleotide sequences, such as (GA)<sub>n</sub> and are widely scattered at many different loci throughout the genome<sup>9</sup> which provide the basis for a PCR-based, multi-allelic, co-dominant genetic marker system<sup>21</sup>.

It has been confirmed that the SSR markers are most informative and appropriate marker system for molecular characterization owing to their high abundance, hypervariability, co-dominance, Mendelian inheritance, technical simplicity, sensitivity, analytical simplicity and are therefore considered as ideal tool for genetic diversity analysis, molecular map construction and gene mapping, construction of fingerprints, analysis of germplasm diversity<sup>11,27,28</sup> including application of heterosis predominantly in identification of species with closer genetic relationship.

Present study was carried out to examine and quantify the level of genetic diversity at molecular level using SSR markers. In this investigation, we used eighty SSRs to generate fingerprints of 40 pigeon pea genotypes of diverse genetic background and to develop unique fingerprint for each genotype. The SSR data was used to evaluate the level of genetic diversity within pigeon pea genotypes, to assess genetic relationships among the varieties and to define whether existing SSR markers provide satisfactory power of resolution to discriminate between varieties for use in commercial evaluation.

## Material and Methods

**Plant material:** In the present study, the genetic materials were obtained from ICRISAT, Patancheru, Hyderabad and field experiment was conducted at Crop Research Centre (CRC), Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut U.P., India. A total of 40 pigeonpea genotypes were used for the genetic diversity analysis. Fresh and healthy seeds of pigeon pea genotypes were sown in the experimental field in a randomized block design with three replications.

Total genomic DNA of each pigeonpea genotype was extracted from the fresh leaves or frozen young leaves using

the cetyl tri-methyl ammonium bromide (CTAB) protocol as described by Murray and Thompson<sup>13</sup>. The DNA was further quantified by spectrophotometer at 260 and 280 nm. The quality and quantity of DNA were checked by agarose gel electrophoresis.

**SSR analysis and gel electrophoresis:** A set of 80 SSR primers described by Burns et al<sup>3</sup> and Odeny et al<sup>15</sup> were selected for the study (Table 2). The primers were custom synthesized by IDT (Integrated DNA Technology), USA and used for the amplification of each of the 40 genotypes. The PCR reaction of isolated genomic DNA was carried out in 10 µl reaction volume containing 1x *Taq* polymerase buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTPs, 0.2 µM of each forward and reverse primer, 1 unit *Taq* polymerase and 25 ng genomic DNA as template. The amplification was carried out in Mastercycler gradient (Eppendorf, Germany) using the following conditions: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, specific annealing temperature for 50 sec, 72°C for 1min and a final extension of 72°C for 5 min.

The SSR-PCR products were separated by 1.5% agarose gel electrophoresis and were visualized by staining with ethidium bromide and trans-illumination under short-wave UV light. The size of the bands was decided by comparing with 100 bp ladder standard marker (Bangalore Genei). The reproducibility of amplification products was confirmed twice for each primer. Amplified products were stored at 5 - 20°C until further use.

**Data analysis:** The electrophoresed gels were scrutinized under ultra violet transilluminator and photographed using Gel Documentation System. All amplification products were scored as present (1) or absent (0) for each of the 40 genotypes with all primers and subjected to produce a binary matrix. Bands with same mobility were treated as identical fragments. The positions of PCR bands were matched with 100 bp ladder as molecular weight standards. Ambiguous bands that could not be clearly distinguished were not scored. The number of polymorphic and monomorphic amplification products generated by every primer and the degree of homology of the examined genotypes were determined for each primer.

Pair wise comparison of genotypes, based on the presence (1) or absence (0) of unique and shared polymorphic products was used to generate Jaccard's coefficient by NT-SYS-pc version 2.2 software<sup>20</sup>. The similarity coefficient was used to construct a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA). Combined analysis was performed by using dendrogram along with Jaccard's coefficient. The polymorphism information content (PIC) value described by Botstein et al<sup>2</sup> and modified by Anderson et al<sup>1</sup> for self-pollinated species was calculated as follows:

$$PIC = 1 - \sum (P_{ij})^2$$

where  $p_i$  equals the frequency of the  $i$ th allele and  $p_j$  the frequency of the allele. Only data from polymorphic loci were used for this analysis.

## Results and Discussion

One of the main purposes of SSR markers in genomic study is the characterization of genetic resources to help genebank management<sup>26</sup> as much as to assess the genetic variability in genotypes used in genetic breeding programs<sup>10</sup>. The degree of relatedness between cultivars and lines of the program can be accurately determined as well as the degree of genetic purity in advanced lines and cultivars particularly in the case of genetic variability used in genetic breeding programs. The DNA of 40 pigeonpea varieties isolated by CTAB method exhibited the values of  $A^{260} / A^{280}$  ratios 1.7 to 1.8. Based on the spectrophotometer readings, the DNA samples were diluted to 25ng/ $\mu$ l for SSR analysis.

In present investigation, primers used varied greatly in their ability to resolve variability among the genotypes. The amplification reactions were reproduced and the bands that were steadily reproduced across amplifications were considered for the analysis. The polymorphic bands formed were efficient in evaluating genetic diversity among the cultivars. A total of 224 bands/ alleles were detected at the loci of 65 microsatellite markers across 40 pigeonpea genotypes of which 189 (84.37%) were polymorphic. The numbers of alleles per locus varied from 2 to 7 alleles with an average of 3.4 alleles per locus and were used for genetic analysis (Table 3).

The highest number of alleles (7) were detected for the marker CZ681974 followed by 6 alleles for CZ445524, CZ445536, CZ681954, CZ682002, CZ682011 and the lowest number of alleles (2) were detected for the markers CZ445545, AJ312887, AJ312892, AJ312895, CZ681920, CZ681929, CZ681937, CZ681938, CZ681940, CZ681955, CZ681960, CZ681965, CZ681966, CZ681977, CZ681989, CZ681990, CZ682005 and CZ682006. Figure 2 (a,b) shows the amplification profiles of the primer CZ681974 detecting 7 alleles across 40 pigeonpea genotypes. No rare alleles, alleles with allelic frequencies less than 0.005 were obtained. This was possibly due to the closeness of the genotypes studied.

The mean allele (3.4 alleles) obtained in our study was comparable with the result reported by Odeny et al<sup>16</sup> detecting 3.1 alleles per SSR locus who used 24 pigeonpea accessions. Singh et al<sup>22</sup> reported the genetic diversity analysis among 16 cultivated pigeonpea genotypes using 22 SSR primers, detecting a total of 46 alleles. The average number of alleles per locus was 2.1 which is evidently lower than our report. In contrast, the mean value obtained from our study is slightly lower than the results observed in previous diversity studies by Sousa et al<sup>25</sup>, Odeny et al<sup>15</sup> and Songok et al<sup>24</sup> who testified an average of 5.1, 4.9 and 8 alleles per locus respectively.

**Polymorphism information content (PIC):** The discrimination power of each locus was estimated by the PIC (Polymorphism Information Content) value. The PIC value determined the polymorphism among varieties for a marker locus used in linkage analysis. The PIC values were not uniform for all of the SSR loci tested which were derived from allelic diversity and frequency among the genotypes. The PIC values for the SSR loci ranged from 0.24 to 0.86 with an average value of 0.50. (Table3). According to the previous reports, PIC values ranged from a low value of 0.17 to a high value of 0.80<sup>15</sup>.

Songok et al<sup>24</sup> found the highest PIC value of 0.65. Singh et al<sup>22</sup> reported that the PIC value ranged from 0.26 to 0.88 with an average of 0.57, little higher than our value. Sousa et al<sup>25</sup> detected a mean PIC value of 0.49 which was similar to our value. The estimated average PIC value (0.50) observed in current study is relatively higher than the average PIC value of 0.41 as reported by Odeny et al<sup>16</sup>. This indicated that genotypes used in the present study were diverse. The highest PIC value (0.86) was observed for primer CZ681974 followed by 0.84 (CZ682002), 0.79 (CZ445524), 0.79 (CZ681927), 0.79 (CZ682011), 0.78 (CZ681941), 0.78 (CZ681954) and 0.78 (CZ681983). The lowest PIC value (0.24) was observed for the Primer CZ682005.

The PIC value of each SSR marker measures their diversity. Molecular markers having PIC values of 0.5 or greater are highly informative for genetic studies and are tremendously useful in distinguishing the polymorphism rate of a marker at a specific locus. The present study revealed that markers CZ681974 would be best for screening 40 pigeonpea genotypes followed by CZ682002, CZ445524, CZ 681927 and so on as evident from their PIC values. Thus, the PIC value specifies that all these primers are highly informative and capable of distinguishing genotypes.

**Genetic relationship and cluster analysis:** Genetic similarity coefficients among 40 pigeonpea genotypes based on the SSR banding patterns were calculated using Jaccard's coefficient analysis. The Jaccard's pairwise similarity coefficient values ranged from 0.45 to 0.93 indicating a wide range of genetic variation present in the pigeonpea genotypes. The highest similarity percentage occurred between two pigeonpea genotypes ICP-3046 and ICP-3049 with a coefficient value of 93% which revealed a high degree of similarity to the extent of 93% existing between them. This was followed by 92% similarity between ICP- 4317 and ICP- 4575, 91% each in ICP- 4167 and ICP- 4307 and ICP- 4167 and ICP-6128 indicated less divergence between them.

Sousa et al<sup>25</sup> reported the genetic distance ranged from 0.09 to 0.62 (average 0.37) showing a low genetic diversity in the pigeonpea genotypes. Earlier Malviya et al<sup>12</sup>, Ratnaparkhe et al<sup>19</sup> and Choudhury et al<sup>4</sup> studied RAPD markers for identification and genetic divergence of pigeonpea genotypes and detected genetic similarity coefficient

ranging from 0.272 to 0.778<sup>12</sup>, 0.7 to 0.9<sup>19</sup> and 0.192 to 0.708<sup>5</sup>. The lowest percentage of similarity occurred between ICP-6815 and ICP-11946 with a coefficient value of 45% which indicate 55 % divergence. Crossing between the genotypes with low similarity coefficient will manifest high heterosis.

Genetic relationships among the accessions were further studied by cluster analysis. The clusters constructed through NTSYS (2.02 pc) presented in the form of dendrogram are shown in fig. 1. A total of 80 SSR primers were used for construction of dendrogram of the 40 pigeonpea genotypes by the UPGMA method (Unweighted Paired Group Method Using Arithmetic Averages) employing sequential, agglomerative hierarchic and non-overlapping clustering (SAHN) to understand the genetic relationships among the pigeonpea genotypes.

The dendrogram of the hierarchical cluster analysis (HCA) separated the 40 genotypes into two main clusters which revealed the proximity of their genetic distance. The diversity within cluster groups suggests a high mutation rate with large amount influencing the phenotype of accessions studied. The first cluster contains 15 cultivars which are divided into two sub-groups A and B at 66% similarity.

Sub-group A comprising of eleven cultivars could be further divided into two branches A1 and A2. The A1 branch consists of 5 cultivars ICP-7, ICP-2577, ICP-7375, ICP-6370 and ICP-6859, where maximum similarity coefficient (0.87) occurred between ICP-2577 and ICP-7375 and minimum similarity coefficient (0.81) occurred between ICP-7 and ICP-2577. The A2 branch consists of 6 cultivars ICP-772, ICP-4029, ICP-6123, ICP-8793, ICP-1126 and

ICP-6739 where maximum similarity coefficient (0.88) occurred between ICP-6123 and ICP-8793 and minimum similarity coefficient (0.79) occurred between ICP-6739 and ICP-6123.

Sub-group B comprised of 4 cultivars ICP-939, ICP-1273, ICP-1156 and ICP-11946 where the maximum similarity coefficient (0.91) occurred between ICP-939 and ICP-1273 and minimum similarity coefficient (0.82) occurred between ICP-11946, ICP-939 and ICP-1156. The second main cluster contains 25 cultivars divided into three major sub-groups C, D and E at 66% similarity.

Sub-group C comprising of 22 cultivars could be further divided into two branches C1 and C2. The C1 branch consists of 6 cultivars ICP- 1071, ICP- 2698, ICP-3451, ICP-6668, ICP-348 and ICP-7221 showing more genetic similarity among themselves in which the maximum similarity coefficient (0.91) occurred between ICP- 1071 and ICP- 2698 and minimum similarity coefficient (0.78) occurred between ICP-7221 and ICP-3451. The C2 branch was the largest group in this study, including 18 cultivars in which maximum similarity coefficient occurred (0.93) between ICP-3046 and ICP-3049 and minimum similarity coefficient (0.76) occurred between ICP-7223 and ICP-2746. ICP- 4715 was the lone member of Cluster D at a similarity coefficient of 0.76.

Sub-group E comprised of only 2 cultivars ICP-11910 and ICP-995 with a similarity value of 0.81 genetically close to each other. Cluster analysis based on genetic similarity values (0.45 to 0.93) provided a significant genetic variation and a clear resolution of relationships among all the 40 pigeonpea genotypes.

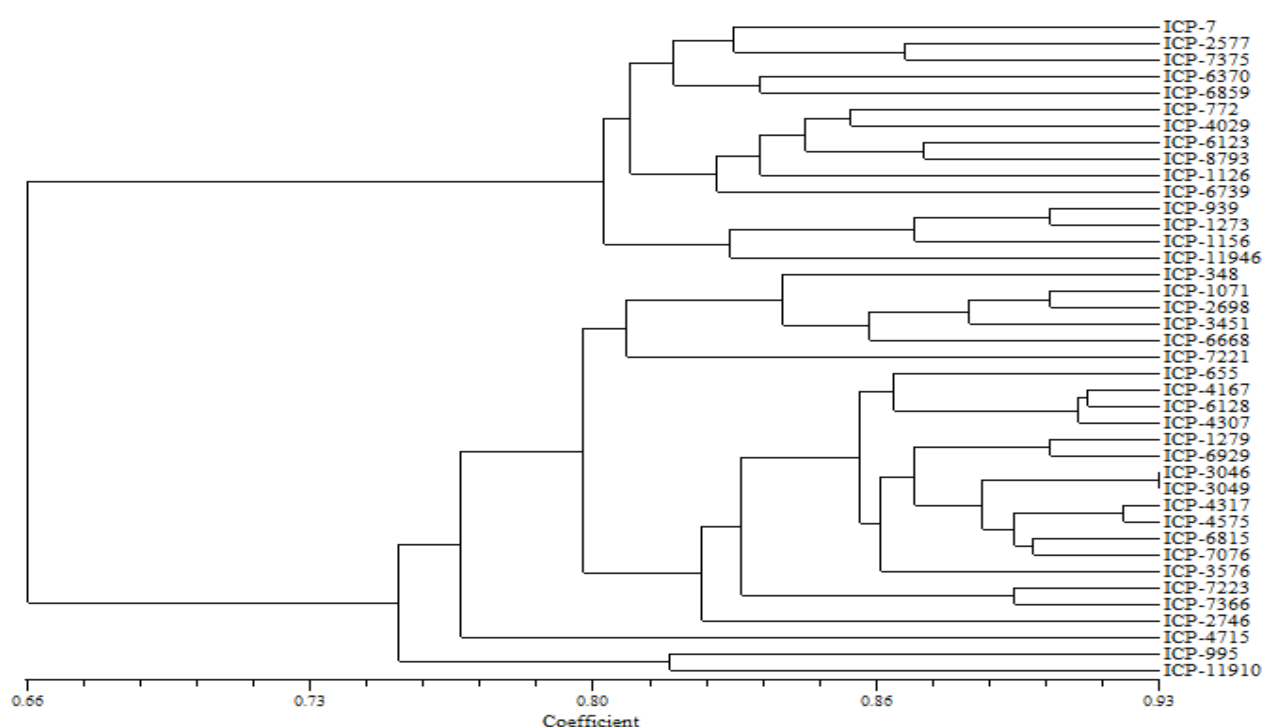
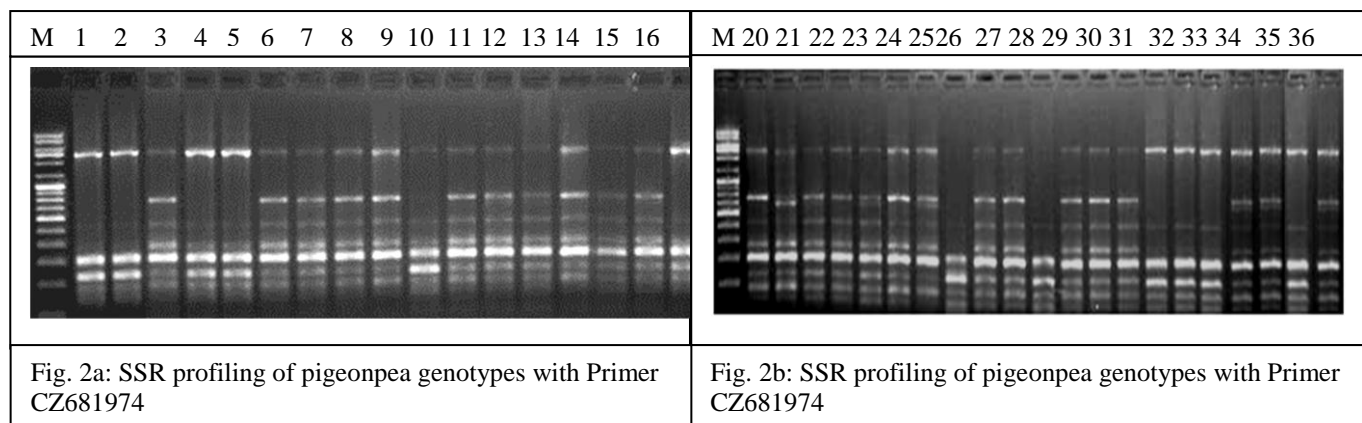
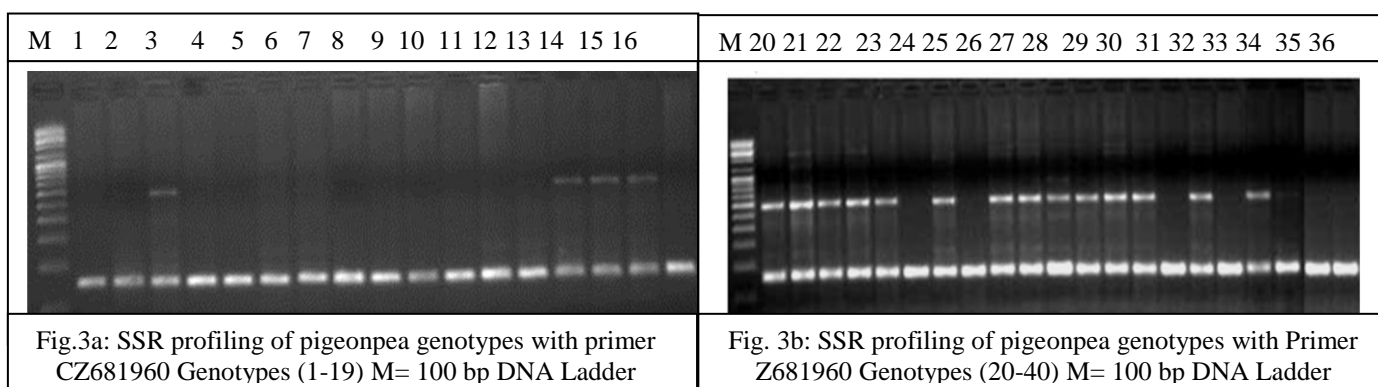


Figure 1: Dendrogram constructed using UPGMA cluster analysis

**Fig. 2: SSR profiling of pigeonpea genotypes****Fig. 3: SSR profiling of pigeonpea genotypes****Table 1**

List of pigeon pea genotypes used in the investigation provided by ICRISAT, Patancheru (Hyderabad)

S. N.	Genotypes	S. N.	Genotypes	S. N.	Genotypes	S. N.	Genotypes
1	ICP-7	11	ICP-1279	21	ICP-4307	31	ICP-6929
2	ICP-348	12	ICP-2577	22	ICP-4317	32	ICP-7223
3	ICP-772	13	ICP-2698	23	ICP-4575	33	ICP-7366
4	ICP-655	14	ICP-2746	24	ICP-4715	34	ICP-8793
5	ICP-939	15	ICP-3046	25	ICP-6123	35	ICP-7221
6	ICP-995	16	ICP-3049	26	ICP-6128	36	ICP-7375
7	ICP-1071	17	ICP-3451	27	ICP-6370	37	ICP-7076
8	ICP-1126	18	ICP-3576	28	ICP-6668	38	ICP-6739
9	ICP-1156	19	ICP-4029	29	ICP-6815	39	ICP-11946
10	ICP-1273	20	ICP-4167	30	ICP-6859	40	ICP-11910

**Table 2**

Sequence of SSRs primers pairs used in amplification in pigeonpea genotypes

S. N.	Primer Code	Primer sequence	S. N.	Primer Code	Primer sequence
1.	CZ681920	F: GCGGGATTCTCTTGCTTAC R: TCACAAAACAATTTGGCACA	41	AJ312889	F:GGAGCTATGTTGGAGGATGA R:CCTTTTTGCATGGGTTGTAT
2.	CZ681922	F: ACACCACCATGCTAAAGAACAAG R: CCAAGCAAGACACGAGTAATCATA	42	AJ312890	F:GACAATTTTGCATGCATTGC R:TTGCAAAAACACTTGGTTGG
3	CZ681925	F: TGCTTCAAGTTGCCTACCAG R: TCAAGGGAGGTGGACTACAAA	43	AJ312891	F: ACAATGCTAGGGAACACCGC R: TACCTTAACCCACAATGGCC
4	CZ681926	F: GTAGAGGAGGTTCCAAATGACATA R: ATCTGTCTGGTGTGTTTAGTGTGCT	44	AJ312892	F: CAACATTTGGACTAAAACTG R: AGGTATCCAATATCCAATTG

5	CZ681927	F: CTCTTGCTTACGCGTGGACT R: CTTTGTCTTTTGCCTGCTT	45	AJ312893	F: TCGTTTTGTAAAGCATTCTTCA R: ACTTGAGGCTGAATGGATTG
6	CZ681929	F: TCACAGAGGACCACACGAAG R: TGGACTAGACATTGCGTGAAG	46	AJ312894	F: CACTTGGTTGGCTCAAGAAC R: GCCAATGAACTCACATCCTTC
7	CZ681930	F: GCGCTAAGGGAAAACAAAAA R: AACTCCCTTGTGTGCATATGGTG	47	AJ312895	F: CCTTCTTAAGGTGAAATGCAAGC R: CATAACAATAAAAGACCTTGAATGC
8	CZ681933	F: AGAGGGAAAGGGAAGAGAAGA R: TCAAGCAACTCCAAGAAATTCA	48	CZ445530	F: CGGGCTTCCTTTTCTTCTCT R: AAAACCCCGAAAACACCATT
9	CZ681934	F: AAGGCTTTTCAACAAATAGGG R: AGAAGAGAAAAAGCATAAACTTCA	49	CZ445525	F: TTCTGGATCCCTTTCATTTTTC R: GACACCCTTCTACCCCATAA
10	CZ681935	F: CATTTATTTCTCTCTGGCATTAC R: CGAGCTGCAAGCATAAACG	50	CZ445522	F: CTTCCCCCAACTAAGATCCA R: GTTCGTTCTCTTTAATTGACTTGC
11	CZ681938	F: TCAGGGGTAAATGCGGTATC R: GAATTGCTTTTGTCTTCTCA	51	CZ445523	F: TTTCTGAGCCATCAGTCG R: AAGCATCAACGTACCAGCAA
12	CZ681940	F: TAAGGAAATGGCTGGGGTTG R: CACATAAATTTGGGGGTTTCG	52	CZ445531	F: TGAATTGCTGAGAGGACGTTT R: CTGTTCCAATTCCACGGTTT
13	CZ681941	F: GGACTTGTTACTGGGGCACT R: AATTCCCATTGGTCATTCG	53	CZ445520	F: CCCATTTAGTGAGGGTTAAT R: GACTACTCCAGGTCAAACACG
14	CZ681946	F: TAATCCCATTCCGTTGTCTG R: CCCAGGAAGAGATGAGACCA	54	CZ445535	F: GTCGGGGCGTGTAAAGTCATA R: CCGAAATAAGGATGGCAAAT
15	CZ681947	F: AGGCTTTCTCCCTTCAATCC R: GCCTTTTCAAACCTTTTCTCACA	55	CZ445536	F: GTCTTTGAGGGACGGAACC R: GGGGCGGGGAAAGTACATA
16	CZ681951	F: ACATGTGTGGCGTAGTGTGA R: GCAAACCGTTCCATAAAAA	56	CZ445538	F: CCAAGAAAAGGTGCTCCAAGT R: TTGCTTCTTTTCTCGCTTGC
17	CZ681954	F: GAGGATTGCACCAAGCAACT R: GCACTGCTGGCCTTACCATA	57	CZ445539	F: TGATAGGACCACAACGACA R: AGCGTTGACTCTCCCTCTT
18	CZ681955	F: TGGGCTGTGATCGATGAAT R: CGACAACAACAACACCGACT	58	CZ445540	F: ACGCTTCTGATGCTGTGTTG R: CATCAGCATCATCGTTACCC
19	CZ682002	F: CAAGGAATCACTTAAAAACCAAGC R: AGATGGCCAAGATTCCACAAC	59	CZ445519	F: GACTCTTCACCTCACACTCATCAC R: ACCTCATACAACAACCTAAGCAC
20	CZ682011	F: AAATTCACCACCATGATCCAA R: TCTTCACTTCCGAGACACAAC	60	CZ445544	F: TACAGCAGCCACATCAAAGC R: TGAACCGTGAAAGTGGGATT
21	CZ681943	F: TGGGCATGGTAGAGGAAGTT R: CGTCATGAAGCAACAGGAGA	61	CZ445553	F: ACCCATTTATTGATTTGGGTA R: CCAAATTTACCCCAAGAAA
22	CZ681964	F: GATAGCACACACACACAACA R: TACCTTAGGGTCACCAACGA	62	CZ445545	F: TCTTCCATTGCATGGTGTT R: GCATGATATGAGATGATGACGA
23	CZ681966	F: AGTCGATGTGGAACATGAGGA R: TGTTGTAAGCCGTGGGTAGG	63	CZ445524	F: CTGGGCCTCTAGCATAGCAA R: AAACCTTCTGGACGCAAAATGA
24	CZ681993	F: ATCATCAGATTCTTCAGCCGTA R: GGTTAGACCAATCCAATCAAGC	64	CZ681957	F: TGTTCGTTTCAAGTGGTCA R: CGACATTTACCCACTCGTTCA
25	CZ681995	F: CACGATTCCATTGGTGGAG R: ACGGTTTCTGGGAGGGTCTA	65	CZ681960	F: TCGTGGGAATGCTCTACAAC R: AACCACAAGTACCCACACC
26	CZ681996	F: CCACAAGTACACCCACACCA R: TTCGTGGGAATGCTCTACAA	66	CZ681962	F: GGGAACTCACCTATATTACCAA R: CACTACCGTCTACAGCCATCTC
27	CZ681923	F: CATCGCCTACAATCATACAAAGA R: TCTTGTCTTTTTCAGTCATCGT	67	CZ681965	F: CTTTGTTCAGAGCGGAGCAT R: TTTTATAGGACATTGGGAAGCA
28	CZ681924	F: ATCGCTTTGCATCCTTATC R: CTCACGTACATTTTCGTTT	68	CZ681969	F: ATCCAGACTTCATAGGGAGATAG R: GTCTAGTCCCAGGTACAAAGAGGT
29	CZ681928	F: TCTTAGCATGCTCTATTTTCGT R: AGTACATTTCAAATCCACACATCC	69	CZ681970	F: CTTCTCCCTGCCTCTTTTCC R: CAAGTGGAGGGGAGTGAAGA
30	CZ681937	F: TGCACAGATTGAAAGTTCC R: CCTCAAGATTCTCTTTCTCTCA	70	CZ681971	F: AAGTTGCCTACTGGGGGTTTC R: AAATAGAGCTGTCAGGGGAGGT
31	CZ682004	F: GCCTTTTCAAACCTTTTCTCA R: CATATGCTTTAAGTGCTTTCCT	71	CZ681973	F: TGGACTACCAAACGCAGACA R: TCGTAGCTGCAGAGCATTTT
32	CZ681963	F: GTTCTTCTTGTGTTGTTGTTG R: AATTCGTGGAGTTCATTGG	72	CZ681974	F: ATCTCCAAAAGTTCCACCA R: CAAAGGAGGATTTCCACCAA

33	CZ682005	F: TGTATGTTTCGTTTAGAGGCTTCC R: GCCCTTTTCACTTTTCTCA	73	CZ681975	F: ACGGTGCCTTGTTGATTGTA R: CGGAACAGGAGGAAAAGGTC
34	CZ682001	F: TCTTTCAGACGCAATGACCTT R: CACTTATTTGTGGGGACCATC	74	CZ681977	F: ACCTTGCTTGTTTCGCTTTT R: AAGGGAGGTGGACTACAAGGA
35	CZ682008	F: AACGATGAAATTCCCAAACG R: TGTTAGATGCTCAACCCAAGG	75	CZ681981	F: ATCATAATCATACATGTCAATGCTA R: GGTTTTATCTTTGTCTCCAATTCTG
36	CZ681998	F: ACAAATCCGGTGACCCATAA R: CCGAGAACAAAAACATTGAACA	76	CZ681983	F: TGGGCATGGTAGAGGAAGTT R: TCAGAAGTCGATGGCAAGTG
37	CZ681958	F: TAGAGCGTTGTCCCTTTTCTG R: TCGAAGGACAACCTCAAGCATT	77	CZ681986	F: TGCTCTAATGGCTAGTTCATCC R: AAACACTCATGGGTAGATTCTCC
38	CZ681967	F: AGGTGCAAAGGAAGCACTAAT R: CAGCTCCACTGTCTTCAACG	78	CZ681989	F: TAGTATGGGCGTGGTAGAGGA R: CGTGACAGAGTCAATCAGAAGC
39	AJ306901	F: AAGGGTTGTATCTCCGCGTG R: GCAAAGCAGCAATCATTTTCG	79	CZ681990	F: CAGGTCTGCTACTGCCATCA R: AGCCCACTTCTGCATCACTC
40	AJ312887	F: CCATAATCCAATCCAAATCC R: AGAAGGCTTTCATGTAACGC	80	CZ682006	F: TGCCTACTAGGGGTTTCGTG R: TGAAGTATCCAGGGAGGTGAG

**Table 3**  
**PIC of SSR loci across various germplasm/ genotypes analyzed**

Primers	Frequency of SSR alleles	No. of alleles	Polymorphic alleles	Percentage polymorphism	PIC
CZ445522	0.42 0.57	2	1	50	0.49
CZ445523	0.24 0.21 0.25 0.29	4	4	100	0.75
CZ445524	0.09 0.06 0.12 0.14 0.24 0.32	6	5	83	0.79
CZ445530	0.19 0.18 0.26 0.36	4	3	75	0.73
CZ445535	0.13 0.18 0.29 0.39	4	3	75	0.71
CZ445536	0.04 0.07 0.08 0.62 0.11 0.05	6	6	100	0.58
CZ445540	0.06 0.08 0.07 0.22 0.55	5	4	80	0.63

CZ445544	0.08 0.12 0.09 0.11 0.58	5	4	80	0.62
CZ445545	0.17 0.82	2	2	100	0.29
AJ312887	0.53 0.46	2	2	100	0.50
AJ312889	0.19 0.20 0.21 0.39	4	4	100	0.72
AJ312891	0.04 0.06 0.08 0.14 0.66	5	5	100	0.53
AJ312892	0.19 0.80	2	2	100	0.32
AJ312893	0.18 0.15 0.23 0.42	4	4	100	0.71
AJ312894	0.21 0.21 0.56	3	2	66	0.60
AJ312895	0.73 0.26	2	2	100	0.40
CZ68196	0.21 0.18 0.26 0.33	4	3	75	0.75
CZ681920	0.45 0.55	2	1	50	0.50
CZ681923	0.26 0.73	2	1	50	0.40
CZ681924	0.09 0.10 0.10 0.10 0.59	5	5	100	0.61
CZ681927	0.12 0.21 0.17 0.22 0.27	5	5	100	0.79
CZ681928	0.21 0.12 0.66	3	2	66	0.51
CZ681929	0.27 0.72	2	1	50	0.41
CZ681930	0.17 0.22 0.6	3	2	66	0.56



CZ681933	0.06 0.13 0.19 0.63	4	4	100	0.55
CZ681937	0.35 0.64	2	2	100	0.47
CZ681938	0.23 0.76	2	1	50	0.37
CZ681940	0.35 0.70	2	2	100	0.38
CZ681941	0.11 0.24 0.18 0.18 0.29	5	5	100	0.78
CZ681943	0.12 0.12 0.12 0.12 0.5	5	5	100	0.69
CZ681947	0.24 0.06 0.06 0.61	4	3	75	0.56
CZ681951	0.15 0.2 0.31 0.26 0.09	5	5	100	0.77
CZ681954	0.01 0.07 0.15 0.16 0.29 0.29	6	4	66	0.78
CZ681955	0.48 0.51	2	1	50	0.51
CZ681957	0.10 0.10 0.15 0.63	4	3	75	0.56
CZ681958	0.16 0.15 0.67	3	2	66	0.50
CZ681960	0.23 0.76	2	1	50	0.37
CZ681963	0.27 0.28 0.47 0.08	4	4	100	0.62
CZ681965	0.55 0.52	2	2	100	0.43
CZ681966	0.52 0.47	2	2	100	0.51
CZ681967	0.13 0.15 0.71	3	2	66	0.46

CZ681970	0.19 0.44 0.41	3	3	100	0.60
CZ681974	0.14 0.11 0.12 0.12 0.16 0.16 0.16	7	4	57	0.86
CZ681975	0.24 0.23 0.25 0.25	4	4	100	0.76
CZ681977	0.26 0.73	2	2	100	0.40
CZ681981	0.12 0.18 0.69	3	3	100	0.48
CZ681983	0.11 0.17 0.23 0.25 0.25	5	5	100	0.78
CZ681986	0.35 0.05 0.6	3	2	66	0.52
CZ681989	0.37 0.62	2	1	50	0.48
CZ681990	0.16 0.83	2	2	100	0.29
CZ681996	0.21 0.78	2	2	100	0.35
CZ682001	0.07 0.19 0.73	3	3	100	0.43
CZ682002	0.15 0.17 0.15 0.16 0.17 0.17	6	3	50	0.84
CZ682004	0.08 0.08 0.33 0.48	4	3	75	0.65
CZ682005	0.13 0.86	2	2	100	0.24
CZ682006	0.22 0.77	2	1	50	0.36
CZ682008	0.30 0.30 0.38	3	2	66	0.68
CZ682011	0.12 0.11 0.03 0.06 0.26 0.34	6	5	83	0.79

## Conclusion

Varying concentration of template DNA revealed that 25ng/μl allowed the maximum number of reproducible bands. The PIC values determined the polymorphism among varieties for a marker locus used in linkage analysis identified most effective markers namely CZ681974, CZ445524, CZ445536, CZ681954, CZ682002 and CZ682011. Further, the lowest percentage of similarity occurred between the genotypes ICP-6815 and ICP-11946 with a coefficient value of 45% indicating 55% divergence. The use of microsatellite markers employed in this study demonstrates the usefulness of these markers for the assessment of genetic diversity and relationships for background selections during back cross breeding programme.

Thus, the genotypes ICP-6815, ICP-11946 along with two other genotypes located in distant clusters ICP-7, ICP-11910 may be utilized in the hybridization programme in order to have a manifested high heterosis during crop improvement programmes.

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